To evaluate the antioxidative activity of the hydrolysates of ovalbumin, the antioxidative activities of the enzymatic extracts were evaluated using three different methodologies: scavenging assays such as superoxide anion, hydroxyl radical, and inhibitory oxidation of linoleic acid in vitro, and the activities of SOD, GSH-Px, CAT and the level of MDA were determined in serum and liver of aged mice induced by G-gal. The results showed that the hydrolysates had a distinctly inhibitory action to superoxide anion \( \text{O}_2^- \) made by alkaline pyrogallic acid, \( \text{HO}^- \) produced by Fenton reaction, the oxidation of linoleic acid in linoleic acid autoxidation system, and presented a positive correlation. The inhibition capacity of hydrolysates against \( \text{O}_2^- \) and \( \text{HO}^- \) were more than 45% and 56% respectively at the concentration 5 mg/mL. And the hydrolysates could significantly (\( p < 0.01 \)) prevent the activities of SOD, GSH-Px, and CAT against reducing and all three concentrations could significantly (\( p < 0.01 \)) decrease the MDA contents in the serum and liver of aged mice induced by G-gal. The antioxidative activity of high concentration was similar to that of control group.

Key Words: ovalbumin, hydrolysates, antioxidative activity, mice

Introduction

Oxidation of biomolecules has been identified as a free radical-mediated process, which causes many unfavorable impacts on food and biological systems. In aerobic organisms, harmful radicals that inevitably form during the metabolism of oxygen are associated with the occurrence of several disease conditions including atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataracts, some neurological disorders and some types of cancer as well as aging. Cellular antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and some food-derived nutritional antioxidants protect tissues from freeradical-mediated oxidative injuries. Therefore, there is a growing interest in identifying antioxidative properties in many natural sources including some dietary protein compounds. Chen et al. has reported that enzymatically derived soybean peptides can act as potent antioxidants against lipid peroxidation and the primary structure of those peptides were critical for their activity. Hu et al. has reported that hydrolysed of casein could raise activities of SOD and GSH-Px in plasma and liver of mice, reduced the level of malondialdehyde (MDA). Recently, a research group reported the antioxidative activity of peptides produced by enzymatic hydrolysis of crude egg white with pepsin.

To evaluate the antioxidative activity of the hydrolysates of egg white proteins, we determined in vitro radical scavenging activities by the auto-oxidation of pyrogallol, the \( \alpha \)-deoxyribose oxidation method, and linoleic acid model system. Furthermore, the effect of hydrolysates on antioxidant enzyme activity and level MDA in serum and liver of mice were detected.

Materials and methods

Preparation of Ovalbumin Hydrolysates

Ovalbumin hydrolysates were prepared by hydrolysis of ovalbumin (grade V, Sigma) with pepsin (P7000, Sigma). The ovalbumin was dissolved in water at a concentration of around 4% and heated at 90°C before enzyme digestion. Pepsin was used sequentially at pH 2 and 37 °C. The hydrolysis reaction was stopped after 6 h by heating to 90°C for 5 min. The hydrolysate fraction with a molecular weight lower than 3,000 Da was lyophilized for activity testing.
Hydroxyl radical scavenging activity assay
Hydroxyl radical scavenging activity was determined according to a slightly modified method of the α-deoxyribose oxidation method. Hydroxyl radical was generated by Fenton reaction in the presence of FeSO₄•7H₂O. A reaction mixture containing each 0.2 mL of 10 mmol/L FeSO₄•EDTA and 0.5 mL of 10 mmol/L α-deoxyribose was mixed with 0.2 mL of the extract solution was added into the reaction mixture until the total volume reached 1.8 mL. Then 0.2 mL of 10 mmol/L H₂O₂ was added into the reaction mixture and incubated at 37°C for 1 h. After incubation, each 1 mL of 3.8% trichloroacetic acid (TCA) and 1.0% thiorbarbituric acid (TBA) were added. Then, the mixture was placed in a boiling water bath for 15 min. Absorbance was measured at 532 nm.

Lipid peroxidation inhibition assay
Linoleic acid was oxidized in a linoleic acid model system to measure the antioxidative activity following the method of Osawa and Namiki with slight modifications. Briefly, the sample was dissolved in 5 mL of 50 mmol/L phosphate buffer (pH 7.0) and added into a mixture of 99.5% ethanol (5 mL) and linoleic acid (0.065 mL) in 0.1 mL of 0.1 mol/L EDTA, 3 mmol/L xanthine, 1.5 mg/mL bovine serum albumin, 0.75 mmol/L NBT, and the serum and homogenates of liver containing SOD. Reaction was initiated by adding 0.1 mL of 56 mU/mL xanthine oxidase. After 30 min of incubation, the reaction was terminated by mixing 6 mmol/L CuCl₂ and was centrifuged at 350 g for 10 min. Absorbance of blue formazan was recorded at 560 nm and 25°C. The relative absorbance was then converted into unit of SOD activity per mL or per mg protein, where one unit of SOD activity was equivalent to the quantity of SOD that caused a 50% reduction in the background rate of NBT reduction.

Superoxide Dismutase Assay
The nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich, which is based on the inhibition of NBT reduction by SOD, was used for the determination of SOD activities. Briefly, 2.5 mL of 0.05 mol sodium carbonate buffer (pH 10) was mixed with 0.1 mL of 3 mmol/L EDTA, 3 mmol/L xanthine, 1.5 mg/mL bovine serum albumin, 0.75 mmol/L NBT, and the serum and homogenates of liver containing SOD. Reaction was initiated by adding 0.1 mL of 56 mU/mL xanthine oxidase. After 30 min of incubation, the reaction was terminated by mixing 6 mmol/L CuCl₂ and was centrifuged at 350 g for 10 min. Absorbance of blue formazan was recorded at 560 nm and 25°C. The relative absorbance was then converted into unit of SOD activity per mL or per mg protein, where one unit of SOD activity was equivalent to the quantity of SOD that caused a 50% reduction in the background rate of NBT reduction.

Animals and experimental design
180 healthy Kunming male mice were selected and divided into 6 groups randomly, one as control group (NC), the other five groups were aged mice group induced by D-galactose (D-gal), ovalbumin group (Ova), low dose of hydrolysates group (L), medium dose of hydrolysates group (M), high dose of hydrolysates group (H), each group with two repeats. The mice were supplemented with 0.25 mL normal saline for NC, and other groups administrated with 150 mg/kg D-gal for 30-d. After that, NC and D-gal groups were administrated with 0.25 mL/d distilled water, and Ova, L, M, H groups were supplemented 10 mg/mL ovalbumin, 4, 10 and 16 mg/mL by 0.25 mL/d respectively 40-d later, all mice were euthanatized, and the activities of SOD, GSH-Px, CAT and the level of MDA were determined. Meanwhile, before mice were euthanatized, all the mice were fasted for 12 h, and blood was sampled from eye, then centrifuged at 3000 g for 10 min to isolate the serum, and stored the serum at -20°C for later use. After death, all livers were collected and washed with cooled saline three times, eliminated fat and connective tissue, dried the surface moisture with filter paper and weighted, then mixed the live and diluted it with cooled saline at an end concentration of 10%, centrifuged at 3000 g, 15 min, the suspension was collected and stored at 4°C for later use.

Catalase Assay
Catalase activity was determined according to the method of Beers et al., by following the decomposition of H₂O₂ at 240 nm and 25°C. Hydrogen peroxide solution (10 mmol/L) in 50 mmol/L potassium phosphate buffer (pH 7.0) with 0.1 mL of the serum and homogenates of liver containing catalase was mixed with inversion, and decrease of absorbance every 30 s over a period of 5 min. Catalase activity was calculated from the change in optical density per minute in the maximum linear rate range using a molar extinction coefficient for NADPH of 6.22×10³ μmol and assuming 2 mol of GSH formed for each mole of NADPH consumed. One unit activity was defined as 1 μmol/L NADPH oxidized per minute.

MDA assay
This assay was used to determine malondialdehyde (MDA) levels as described by Ohkawa et al., by following the decomposition of H₂O₂ at 240 nm and 25°C. Hydrogen peroxide solution (10 mmol/L) in 50 mmol/L potassium phosphate buffer (pH 7.0) with 0.1 mL of the serum or homogenates of liver was added into 50 μL of 8.1% sodium dodecyl sulfate (SDS), vortexed, and was incubated for 10 minutes at room temperature.
20% acetic acid (pH 3.5) and 375 μL of thiobarbituric acid (0.6%) were added and placed in a boiling water bath for 5 min. The samples were allowed to cool at room temperature, then 1.25 mL of butanol:pyridine (15:1) was added, vortexed and centrifuged at 1000 g for 5 minutes. 500 μL of the colored layer was measured at 532 nm using 1,1,3,3-tetraethoxy propane as a standard.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.

**Results and discussion**

**Antioxidative activities of ovalbumin hydrolysates in vivo**

Antioxidative activities of ovalbumin hydrolysates in vivo were investigated by aged mice model, which was induced by injected daily with D-galactos. The activities of SOD, GHS-Px, CAT and concentration of MDA in treatment group mice were shown in Table 1. From table 1 we can see, the SOD, GHS-Px, and CAT activities decreased significantly, and the MDA level increased in the liver and serum of aged mice. However, hydrolysates could significantly prevent the SOD activity against reducing in the serum and liver of the mice that induced with D-gal, which indicates that hen egg ovalbumin hydrolysates processing antiaging function. In this experiment, of all three concentrations groups, H group possessed higher SOD level in serum and liver, but serum is slightly lower than liver, it suggests that hydrolysates play an important role in the antioxidative defense status of serum, but increases it in liver, which consisted with the other study in rat with fish protein. Also, this suggests that the antioxidative of hydrolysates is dose-dependent.

**Statistical analysis**

Values were presented as means ± standard deviation (SD) when appropriate. Differences between groups were evaluated by one-way ANOVA, followed by a least-significant difference (LSD). All analyses were performed with the statistical software DPS8.0.
Radical scavenging activity

Free radicals with the major species of reactive oxygen species (ROS) are unstable, and react readily with other groups or substances in the body, resulting in cell damaging and human disease. Especially, the chemical activity of hydroxyl radical is the strongest among the ROS. It easily reacts with biomolecules such as amino acids, proteins, and DNA. Therefore, the removal of hydroxyl radical and superoxide anion is probably one of the most effective defences of a living body against various diseases. The resultant hydrolysates were tested for their scavenging activities on two different radicals, superoxide, and hydroxyl radical using the pyrogallol auto-oxidation system, and Fenton reaction (Fe²⁺ + H₂O₂ → Fe³⁺ + O²⁻ + HO•), respectively. We could observe that ovalbumin hydrolysates exert potential scavenging effects on two radicals in different capacities. Figure 1 presents the inhibitory rate of the superoxide productivity in concentrations of hydrolysates. The hydrolysates of ovalbumin exhibited antioxidant activity, and the antioxidant activity increased with increasing concentrations, and more significantly (p < 0.01) than the antioxidant activity of ovalbumin with the same concentration. Superoxide anion (O₂⁻) is formed in viable cells during several biochemical reactions and its effect can be magnified because it produces other types of free radicals and oxidizing agent that can induce cell damage. In our research, the inhibitory capacity of hydrolysates against O₂⁻ was more than 70% at the concentration of 5 mg/mL.

Figure 1. Effects of hydrolysates from ovalbumin on inhibited the auto-oxidation of pyrogallol

Concentration (mg/mL)  

Concentration (mg/mL)  

Concentration (mg/mL)  

Figure 2. Effects of hydrolysates from ovalbumin on hydroxyl radical scavenging activity

Figure 3. Effects of hydrolysates from ovalbumin on antioxidative activities in linoleic acid autoxidation system

higher than that of liver, which indicated that ovalbumin hydrolysates functioned mainly in serum. This differs with the anti-oxidation activity of VE, which functions indirectly through serum or the oxidases in the tissue. Hen egg ovalbumin hydrolysates could significantly inhibit the GSH-Px activity reducing in the serum and liver of the mice that induced with D-gal, which indicates that hydrolysates processing antiaging function.

H₂O₂ is an important semi-finished product during the free radical metabolism. In common, superoxide anion free radicals could be catalyzed by SOD and produce H₂O, and H₂O₂ could be cleared by catalase immediately, and when the catalase is insufficiency, H₂O₂ can penetrate cellular membrane and damage the cell structure. High activity of SOD and CAT can prevent the lipid peroxidation, main the integration of the cellular membrane. Activities of CAT in experimental groups were higher than that of D-gal group both in serum and liver, and H group was the highest. Elevation of CAT and SOD could be an indication of increased antioxidant protection of the tissues.

Aging causes prominent oxidative damage to various cellular components, especially in tissues with high oxidative capacity. Therefore, it is not surprising that lipid peroxidation and protein oxidation in the aged heart and oxidative muscle fibers were intensified, as demonstrated by the higher levels of MDA and carbonyl formation. MDA is the superoxide products of polyunsaturated fatty acids in the body induced by O₂⁻, and its level can reflect the free radical contents, thereby the damaged degree can be deduced indirectly. In the present study, significantly lower serum and liver concentrations of MDA were observed in mice receiving hydrolysates supplementation. The plausible reason for this is that hydrolysates attenuate free radical production. It may directly prevent the production of free radicals or it may facilitate the scavenging of free radicals.

Inhibitory effects of ovalbumin hydrolysates on lipid peroxidation

To assess the inhibitory effects of Ovalbumin hydrolysates on lipid peroxidation, linoleic acid was oxidized using a
linoeleic acid model system where transition metal ion, Fe$^{2+}$, accelerated lipid peroxidation (Fig 3). Several studies have already observed that some hydrolysates derived from different protein sources possess antioxidative effects. As observed in this research, the hydrolysates could retard lipid peroxidation efficiently than that of control. Among concentrations of hydrolysates, the concentration of 5 mg/mL, and 10 mg/mL exhibited the highest activities and their antioxidative patterns were closer to that of α-tocopherol with 0.2 mg/mL concentration during 7 days of oxidative reaction.

In summary, ovalbumin hydrolysates supplementation mice decreased oxidant production and age-related oxidative damage. Elevated SOD and GPX activities may partially explain these protective effects in mice, whereas the mechanism benefiting the aged mice are currently unknown. Future studies should be conducted to verify this effect and elucidating the mechanisms underlying the protection.

References